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*Research Article*

EXPERIMENTAL MODEL

# Immune Characterization of Wild-Caught *Rattus norvegicus* Suggests Diversity of Immune Activity in Biome-Normal Environments

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**Abstract** Biome depletion, or loss of co-evolved constituents within the ecosystem of the human body, has become the leading suspect in epidemics of allergic, inflammatory and autoimmune diseases associated with post-industrial culture. Immunity in wild and laboratory rats has been used as a model for immunity in biome-normal and biome depleted environments, respectively. In this study, the ranges of numerous immune parameters (such as cytokine production and cell surface marker expression) in 8 wild rats overlapped with the ranges found in 7 laboratory rats. However, considering a number of parameters simultaneously as an "expression index" revealed a substantially greater range of immune activity in the wild compared with laboratory rats. These findings are consistent with the intuitive idea that the immune system is inherently malleable or flexible within environments of evolutionary adaptedness, and might suggest that biome reconstitution as an effective therapy could be achieved in a variety of ways.

**Keywords** allergy; autoimmunity; biome depletion; immunity; laboratory rats; neuroinflammatory disease; wild rats

## 1 Introduction

Autoimmune diseases and allergic disorders occur much more frequently in countries that enjoy the widespread use of modern medical care, sanitation practices, and water treatment technology [4, 10, 34]. Autoimmune disease and allergy affect as much as 6% and 12%, respectively, of the population in post-industrial countries [22], with more than 40% (2 out of every 5) of the children in the US currently suffering from non-infectious, chronic illnesses [7]. It is now well established based on a wide range of direct and indirect evidence that depletion of co-evolved components (e.g., helminths) from the "ecosystem of the human body" as a result of modern medicine and sanitation practices contributes to immune-induced pathology in post-industrial culture [9, 24, 37, 38, 39]. A complex range of interactions [20] between vertebrates and components of their environment over millennia drove the evolution of a vastly complex co-dependency between immune function and those components [9]. The loss of these components in post-industrial society has been termed "biome depletion," where the biome refers to the ecosystem of the human body; and depleted components are not limited to helminths, but can include regular exposure to sunlight lost due to indoor working conditions and/or species of the microbiome killed by antibiotics. In this context, the human biome contains numerous components, including the microbiome and helminths, with the immune system serving as the interface between the human organism and other species associated with the human biome. This concept of biome depletion-associated immune disease applies to a wide range of diseases [9, 38], including allergy, autoimmunity, inflammatory bowel disease, neuroinflammatory disorders potentially including autism, and atherosclerosis, pointing toward the critical importance of understanding the immune system present in pre-industrial environments. It is this immune system, one resistant to the plague of post-industrial immune disease, that must be re-established at least in some regards by modern medicine if patients are to be effectively treated [9].

The link between biome depletion and disease is likely complex, and probably involves multiple elements. First, some components of the biome (e.g., helminths) produce compounds which directly down-regulate host immunity [25]. Loss of these components from the biome undoubtedly increases immune sensitivity and

the magnitude of the immune response. A second potential factor is less specific and involves the fact that the immune system has a limited capacity to respond to stimulus: loss of components from the biome has left some compartments of the immune system (e.g., those that produce IgE) relatively unused, with potentially a much greater capacity to respond to stimulation than would be observed in individuals with a normal biome. Thirdly, a number of studies in laboratory animals suggest that attempts to normalize the biome (i.e., reintroduce helminths) stimulate the development of regulatory networks [42], which limit the threshold and severity of immune responses. Thus, biome depletion probably results in a lack of development of regulatory immune networks, which is expected to contribute to a hyper-responsive immune disposition. However, details regarding the effects of biome depletion on immunity are lacking, in large part because studies aimed at evaluating the immunity of organisms with a "normal" biome (i.e., a biome unmanipulated by modern sanitation and health care practices) are limited in number and in scope.

The immune system of wild-caught rats has been used as a model for human immune systems in the presence of a normal biome [18,29]. The rat offers several advantages over most other animals. First, reagents are widely available to characterize immune components in the animals, separating them from all other species except for mice, humans, and some non-human primates. Second, animals are readily available both living under natural (wild) conditions and living in laboratory conditions with biomes depleted by modern medical and sanitation practices. These two factors combined separate rats from all other species with the exception of *Mus musculus* (house mice/laboratory mice). Further, rats rather than mice generally provide the better model for study of inflammation associated neurological and behavioral dysfunction, which may be important in the pathogenesis of such diseases as autism [5,6] and Alzheimer's disease [8]. Since these diseases may be associated with biome depletion [5,6,9], the wild rat is an invaluable source of information potentially related to human health.

Previous work comparing lab rats with wild-caught rats showed that wild rats have substantially greater levels of autoreactive, polyreactive IgG, but not autoreactive, polyreactive IgM in their serum than do laboratory rats, both on a quantitative and qualitative basis [18]. Increased levels of serum IgG and IgE were observed in wild rats compared to laboratory rats, with the effect being most pronounced for IgE levels. Further, wild rats had higher intrinsic levels of both TH1 and TH2-associated IgG subclasses than did lab rats. The presence in wild rats of increased intrinsic, presumably protective, non-pathogenic responses similar to both autoimmune (autoreactive IgG, TH1-associated) and allergic (IgE, TH2-associated) reactions as well as increased levels of TH1-associated and TH2-associated IgG subclasses points toward a generally increased stimulation of the immune system in these animals rather than a shift in the nature of the immunoreactivity. Thus, at least to the extent that feedback inhibition is a controlling element of immunoreactivity, biome depletion probably lowers profoundly the threshold of both TH1 and TH2 immune responses more so than the balance between the two responses.

Additional studies showed that lab rat T cells but not wild rat T cells proliferated and upregulated the activation markers CD25 and CD134 in response to mitogen in culture [29]. Further, splenocytes from wild rats produced almost 10-fold less IL-2 and TNF-$\alpha$ in response to mitogen than did splenocytes from laboratory rats. In addition, mitogen stimulation resulted in an almost 100-fold greater production of IL-4 in wild rat splenocytes compared to lab rat splenocytes. However, it was concluded that the cytokine release profile of splenocytes from laboratory versus wild rats does not clearly point toward a difference in propensity for either TH1 or TH2 responses between the two groups of rats [29]. The response of splenocytes to mitogen is not restricted to particular strains of lab rat or even to particular species, thus serving as an excellent marker for immune status that is not expected to depend on genetic differences between wild rodents and laboratory rodents.

Interestingly, wild rat splenic T cells cultured for 2 days without mitogen stimulation expressed substantially more of the activation marker CD134 but less of the activation marker CD25 ($P = .0001$) than did identically treated splenic T cells from laboratory animals. [29], suggesting that the phenotype of T cells may differ strongly in biome depleted versus biome normal populations.

Another recently published study [45] described the phenotype of lymphocytes derived from the thymus, spleen, and blood of wild-caught rats, and compared that with results of side-by-side analyses using cells derived from laboratory raised rats and, when possible, previously published values. The results pointed toward a variety of differences in the immune system of hygienic animals that might result from biome depletion, including differences in expression of markers involved in complement regulation, adhesion, signaling, and maturation. These changes suggested increased complement regulation and decreased sensitivity in wild-caught rats compared to laboratory rats, and point toward complex differences between the maturation of T cells in the two groups. However, perhaps surprisingly, the results did not point toward the number of regulatory T cells as being of substantial interest in this regard. Consistent with this view, studies in wild and laboratory mice indicate that the abundance of T regulatory cells is not the central difference between biome-normal and biome-depleted populations [1].

In this study, we continue the detailed characterization of the immune system of wild rats. Immune components from

the bronchoalveolar lavage fluid (BALF) and components of innate immunity in the periphery and spleen were characterized in wild-caught rats. These results were compared to results obtained using laboratory rats, and, when possible, to values in the literature. Particular attention was given to variation seen in the wild population, and the potential implications of this variation for medicine.

Since we could not determine *a priori* which immune parameters might be important in wild immunity, a very detailed analysis of the immune parameters in the animals captured was undertaken. The experimental design we employed in this study involved samples taken from spleen, blood, thymus, and lung, and involved analysis of more than 170 immune parameters, comprising one of the most comprehensive immune surveys reported to date. We previously reported more than 80 of these parameters [45] in the specimens collected (see summary above), and the present report presents an additional 97 derived from the same specimens. The same 170-plus parameters were also analyzed in laboratory rats in order to ensure that the analytical methods were functioning properly, and thus that any unexpected results found in the wild rats reflect the immune status of wild rats and not technical problems. The simultaneous analysis of both wild and laboratory rats also provides a basis for comparison of the two groups.

Due to the very high costs of detailed immunological analyses and the lack of any major initiatives by funding agencies to provide resources for the assessment of immunity in wild animals, initial studies in this field will necessarily be limited in either the number of immune parameters analyzed or in the number of animals assessed. The study we undertook was limited to $N = 8$ wild rats and $N = 7$ laboratory rats. The small number of animals used poses a central limitation; the results may not reflect the immune status of wild rats or laboratory rats in the general population. However, the immune status of laboratory rats has been described previously, and thus the literature can be used as a basis for comparison for at least some immune parameters in laboratory rats. Further, the immune statuses found in wild rats [29] and in humans living in pre-industrial cultures [11] share some similarities, and reflect a condition not found in laboratory mice, laboratory rats, or humans in post-industrial cultures. Thus, immune statuses in the wild may be so different than immune statuses in biome depleted environments that differences transcend differences between species and are thus largely independent of genetics. Nevertheless, the small number of samples examined in this study mandates that future studies are needed to determine the applicability of the results to the general population.

*The study we conducted was not designed to evaluate the effect of various factors on the immune parameters of wild rats. Rather, the study asks what are the general features of immunity in a sample of wild rats, and how do those features differ from what is generally seen in laboratory rats.* Although more traditional studies with larger samples sizes and fewer immune parameters have merit, the results obtained by probing a wide range of immune parameters in a limited sample size are worthwhile and have profound implications for the field of evolutionary medicine.

## 2 Materials and methods

### 2.1 Study design

The study design incorporated a uniquely broad characterization of the immune system, as described below, in order to identify a spectrum of differences between wild and laboratory rats. The number of animals evaluated ($N = 8$ wild animals and 7 laboratory animals) was sufficient to detect differences between wild and laboratory rats which entail a relatively large ratio of difference between the means/standard deviation. A post-hoc analysis of the data revealed that differences were significant ($P < .05$) when the difference between the means of the wild and laboratory animals was approximately 1.2-fold greater than the average standard deviation of the two groups. By the same token, differences were not statistically significant when the difference between the means was less than 1.2-fold greater than the average standard deviation of the two groups.

Data obtained using wild-rat derived samples was compared with data obtained in side-by-side studies with laboratory rodents, as described below. One potential approach to providing a more robust comparison between wild and laboratory rats would be to consider previously published data obtained using laboratory rats in the analysis. This approach is particularly attractive, since studies using a wide range of laboratory rat strains might be considered. Unfortunately, this idea is of limited utility since a broad characterization of the laboratory rat immune system is generally lacking, and since only recently have specific germ free (barrier) facilities become the standard for laboratory studies. Thus, data obtained prior to the early to mid 1990s may utilize animals that have a biome not as depleted as the rodent biomes currently found in standard laboratory rodent housing.

### 2.2 Animals

The animals used in this study have been described previously [45], and some aspects of the immunity of the animals used in this study have been described previously [45]. In particular, the spleenic, peripheral, and thymic lymphocytes, as well as the associated cytokines in the peripheral blood, have been previously evaluated and reported. This report does not include those data, which have been summarized in Section 1. A brief summary of the animals used in this study is as follows: all studies were approved by the Duke University Animal Care and Use Committee. Wild rats ($N = 8$) were caught in live traps within a two-month period, between December 3rd, 2008

and February 3rd, 2009. Captured animals were euthanized by $CO_2$ inhalation within 12 hours of capture. The wild rats were obtained from food processing facilities ($N = 5$) or from urban residential areas ($N = 3$) in North Carolina. Wild rats ranged in weight from 186 g to 407 g, and both males ($N = 3$) and females ($N = 5$) were used in the study. An analysis of fecal samples from the wild rats by PCR (performed by Charles River Research Animal Diagnostic Services, Wilmington, MA, USA) revealed a wide range of potential pathogens, including rat parvo virus (strain RMV), various bacteria (e.g., Beta *Strep* group B, *Camphylocacter jejuni*, *Corynebacterium kutscheri*, *Helicobacter* genus, *Klebsiella pneumonia*, *Staphylococcus aureus*), and the protozoal parasite *Spironucleus muris*. In addition, five out of 8 of the wild rats evaluated had either active colonization with liver flukes (at least one live organism recovered from the liver) or evidence of past liver fluke colonization (focal scarring of the liver).

Male laboratory rats were obtained from Harlan Laboratories (Indianapolis, IN, USA) (WKY, $N = 6$ and Fischer 344, $N = 1$) and euthanized by $CO_2$ inhalation. Those animals ranged in weight from 177 g to 401 g, and were used for comparison with the wild rats.

### 2.3 Blood and tissue processing

Blood and processing of the spleen were performed as described previously [45]. The first 5 mL of blood was drawn from the inferior vena cava into evacuated sodium citrate blood collection tubes (Beckton Dickinson, Franklin Lakes, NJ, USA). Blood was then diluted with 5 mL phosphate buffered saline, pH 7.2 (PBS) and layered over 3 mL of Lymphocyte Separation Media (MP Biomedicals, Solon, OH, USA). Blood was spun at 1930 g for 18 minutes. Plasma and platelets were removed and cells were washed with 30 mL PBS, pelleted by centrifugation at 480 g for 5 minutes and used for immediate flow cytometric analysis. After the first 5 mL was drawn, additional blood was drawn into clot activator blood collection tubes (Becton Dickenson), allowed to clot at room temperature, and centrifuged at 1930 g for 10 minutes to collect sera. Sera were aliquoted and stored at $-80\,^{\circ}$C until assayed.

Spleens were removed and cells were expressed using light pressure and PBS as follows. Small holes were made in one end of the spleen with a 22GA needle. A filled 10cc syringe with a 22GA needle was inserted into the opposite end of the spleen and PBS was slowly introduced. The syringe was refilled and the process was repeated until most cells were expressed and spleens were whitish in color. Tissue and cells were kept at 4 °C for the entire procedure. Cells were centrifuged at 480 g for 5 minutes and used for immediate flow cytometric analysis.

The descending aorta was opened, and blood was flushed from the lungs using about 30 mL of saline infused into the right ventricle via a 14 guage angiocatheter. The bronchus was dissected free and 3 mL of saline with 1 tablet of Complete MiniProtease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN, USA) for each 5 mL of saline was flushed into the lungs via a second 14 guage angiocatheter fastened to the bronchus. After removing approximately 2 mL of the BALF, another 3 mL of saline with protease inhibitors was infused into the lungs. Following removal of another 2 mL of fluid, a final 1 mL of saline with protease inhibitor was added and removed. The BALF from all three washes was then pooled and stored on ice prior to use. Pooled washes from BALF were centrifuged at 480 g for 5 minutes at 4 °C. Pelleted cells were used for immediate flow cytometric analysis, and the supernatant was centrifuged a second time at 600 g for 8 minutes at 4 °C to remove all cells and debris. The resulting supernatant was flash frozen in liquid nitrogen and stored at $-80\,^{\circ}$C until needed for cytokine and immunoglobulin assays.

Following removal of the organs, rats were weighed on a scale to the nearest gram, and the weight of the rats prior to removal of the organs and blood was calculated for each animal based on the average percentage of weight loss upon removal of the blood and organs, which had been previously determined.

### 2.4 Flow cytometry

All cell types were kept at 4 °C and were processed and stained identically. Cell pellets were resuspended in 0.15 M ammonium chloride and 10 mM potassium carbonate and incubated for 2–5 minutes to lyse red blood cells. PBS (35 mL) was added to halt lysis and suspensions were centrifuged at 480 g for 5 minutes. Cell pellets were resuspended in 100 microliters of rat serum, incubated for 15 minutes to block non-specific antibody binding, and then washed through a 35 micron cell strainer with PBS. Cells that were stained with mouse IgM antibodies were also pre-blocked with purified mouse IgM (G155-228, BD Biosciences, San Jose, CA, USA) for 15 minutes. Cells were washed with PBS and centrifuged at 480 g for 5 minutes. Cell pellets were resuspended in PBS and incubated for 20 minutes with LIVE/DEAD Fixable Violet Dead Cell Stain (Molecular Probes, Eugene, OR, USA). Cells were washed with PBS with 1% bovine serum albumin (BSA) and centrifuged at 480 g for 5 minutes. Cells were resuspended in PBS with 1% BSA and stained for the markers listed below.

Antibodies were obtained from BD Biosciences unless otherwise noted: PE anti-CD3 (G4.18), APC anti-CD3 (1F4), PE anti-CD4, PE-CY5 anti-CD4 and APC anti-CD4 (OX-35), PerCP anti-CD8a and Biotin anti-CD8a (OX-8), Biotin anti-CD11b/c (OX-42, AbD Serotec, Raleigh, NC), PE anti-CD25 (OX-39), PE anti-CD28 (JJ319), Alexa Fluor 488 anti-CD45RA (B cell only marker) (OX-33, AbD

Serotec), FITC anti-CD59 (TH9), PE anti-CD62L (HRL1), PE anti-CD86 (24F), Alexa Fluor 488 anti-CD90 (OX-7, AbD Serotec), FITC anti-CD134 (OX-40), Alexa Fluor 647 anti-CD161a (10/78, AbD Serotec), FITC anti-CD172a (ED9, AbD Serotec), PE anti-CD200R (OX-2 receptor) (OX-102, AbD Serotec), FITC anti-Granulocytes (HIS48) and PerCP anti-MHCII (RT1B, I-A) (OX-6).

Following primary antibody staining, some cells were fixed and permeabilized for intracellular staining of Alexa Fluor 488 anti-FoxP3 (FJK-16s, eBioScience Inc., San Deigo, CA, USA) with BD Cytofix/Cytoperm following manufacturer's directions (BD Biosciences). Biotin labeled cells were stained with 2 mg/mL APC-Alexa Fluor 750 streptavidin (Invitrogen Corp., Carlsbad, CA, USA). Streptavidin without biotin labeled primary antibodies and properly labeled isotype antibodies were used as controls and fluorescence minus one controls were used for FoxP3 gating.

Cells were washed and fixed with PBS with 1% BSA and 0.8% paraformaldehyde. Cells were analyzed within 24 hours in the Duke Human Vaccine Institute Flow Cytometry Core Facility that is supported by the National Institutes of Health award AI-51445 using an LSR II cytometer and FlowJo software.

### 2.5 Cytokine, chemokine, and antibody levels

Sera and the supernatant from the bronchoalveolar lavage fluid were analyzed for multiple analytes using Procarta Cytokine Assay kits (Affymetrix, Fremont, CA, USA) except for TGF-beta 1, 2, and 3 and soluble CD62L (L-selectin) which were analyzed with Fluorokine Multiplex Kits (R&D Systems, Minneapolis, MN, USA). Samples were acid activated for TGF measurements and all assays were run as directed by the manufacturer. Assays were analyzed using a BioPlex reader (Bio-Rad Laboratories, Hercules, CA, USA).

Relative concentrations of various antibodies in the supernatant of the bronchoalveolar lavage fluid were analyzed by ELISA using the method previously described for quantification of antibody levels in rat sera [18]. Relative levels of antibodies in the lavage fluid were normalized with respect to levels of albumin in the fluid. Relative levels of albumin in the supernatant of the bronchoalveolar lavage fluid were analyzed on 96-well Maxisorp plates (Nunc, Denmark) using a Rat Albumin ELISA Quantitation Set (Bethyl Laboratories, Inc., Montgomery, TX, USA) according to the instructions provided by Bethyl Laboratories, Inc.

### 2.6 Statistical analysis

A 2-way ANOVA was used for evaluation of the data. For this evaluation, the rat type (wild or laboratory) was used as one variable in all analyses, with the immune parameters measured as the second variable. A separate analysis was run for each of the following 9 parameters: phenotypes of small cell populations in the bronchoalveolar lavage fluid; phenotypes of large cell populations in the bronchoalveolar lavage fluid; cytokines and chemokines in the bronchoalveolar lavage fluid; immunoglobulin levels in the bronchoalveolar lavage fluid; phenotypes of monocytes in the periphery; phenotypes of granulocytes in the periphery; phenotypes of monocytes in the spleen; phenotypes of granulocytes in the spleen; cytokine and chemokine levels in the periphery. An unpaired, two-tailed $t$-test was utilized for post-hoc comparisons to assess differences in means, an F-test was utilized for assessment of differences in variances, and a Bonferroni correction was applied to account for the multiple variables analyzed [32]. GraphPad Prism, Versions 3.0 or 5.0, were utilized for all statistical calculations. An alpha of 0.05 was taken to be significant, and the means $\pm$ standard errors are reported.

## 3 Results

### 3.1 Overall differences between immune parameters in wild and laboratory rodents

The 2-way ANOVA revealed that all data sets except one (phenotypes of spleenic monocytes) contained significant differences between wild and laboratory rats, either dependent or independent of interaction with the second parameter. Of the 9 data sets (parameters) analyzed (described in Section 2), five demonstrated highly significant ($P > .0001$) differences between wild and lab rats by the 2-way ANOVA, a result that is probably not surprising given the substantial differences between wild and laboratory rats. All data were evaluated with a post-hoc unpaired $t$-test using a Bonferroni correction, as described in the tables. Using this approach, 40 out of 105 immune parameters assessed were found to be significantly different between wild and laboratory rats (indicated by bold font in Tables 1–6), with 16 of those still significant following the Bonferroni correction (indicated by the asterisks in Tables 1–6).

### 3.2 B cell populations and immunoglobulin production in the bronchoalveolar lavage fluid of wild and laboratory rats

The results of a broad analysis by flow cytometry of cellular immunity in the bronchoalveolar lavage fluid of lab and wild rats are shown in Table 1. Although the relative number of lymphocytes as compared to macrophages in the laboratory rats is substantiated by previous studies using male Sprague-Dawley rats [48], details concerning the phenotypes of lymphocytes in the bronchoalveolar lavage fluid of rodents are generally not established [35]. The bronchoalveolar lavage fluid from wild rats tended to have relatively more small cell types (T cells, B cells, NK cells, and monocytes), whereas laboratory rats tended to have more larger cell types (macrophages and granulocytes). This difference was apparently accounted for in the extensive numbers of B cells in the bronchoalveolar lavage fluid of the wild rats.

**Table 1:** Phenotypes of cells from bronchoalveolar lavage fluid of laboratory and wild rats.

| Cell population | Lab rat (%) | Wild rat (%) | $P$-value mean | $P$-value variance |
|---|---|---|---|---|
| **Small cells** | $4.64 \pm 1.33$ | $11.6 \pm 2.33$ | **.0203** | .2015 |
| T cells | $20.2 \pm 1.53$ | $20.9 \pm 5.94$ | .9017 | **.0044** |
| $CD4^+CD8^-$ | $72.4 \pm 2.80$ | [a]$76.0 \pm 5.93$ | .5946 | .0896 |
| $CD25^+$ | $82.0 \pm 4.91$ | [a]$75.2 \pm 3.56$ | .3021 | .4004 |
| $FoxP3^+$ | $12.1 \pm 4.55$ | $15.2 \pm 4.63$ | .6453 | .9169 |
| $CD4^-CD8^+$ | $11.8 \pm 0.85$ | $16.1 \pm 5.42$ | .4528 | **.0003**$^*$ |
| $CD4^+CD8^+$ | $2.69 \pm 0.66$ | $4.23 \pm 1.58$ | .3857 | .0537 |
| $CD28^+$ | $90.6 \pm 1.52$ | $78.7 \pm 4.63$ | **.0308** | **.0158** |
| $CD59^+$ | $14.2 \pm 3.73$ | $14.9 \pm 3.18$ | .8828 | .7081 |
| $CD62L^+$ | $25.6 \pm 4.57$ | $12.6 \pm 2.02$ | **.0235** | .0668 |
| $CD86^+$ | $31.0 \pm 3.77$ | $28.5 \pm 3.83$ | .6481 | .9699 |
| $CD90^+$ | $8.36 \pm 2.08$ | $12.4 \pm 2.28$ | .2121 | .8288 |
| $CD161a^+$ | $31.0 \pm 7.40$ | $35.3 \pm 6.25$ | .6664 | .6902 |
| $CD134^+$ | $7.29 \pm 1.47$ | $12.0 \pm 3.22$ | .2125 | .0784 |
| $CD200R^+$ | $3.01 \pm 0.89$ | $6.70 \pm 1.89$ | .1026 | .0920 |
| $MHCII^+$ | $12.9 \pm 2.09$ | $32.9 \pm 8.10$ | **.0339** | **.0044** |
| NK-T cells | $1.75 \pm 0.41$ | $1.58 \pm 0.39$ | .7725 | .9278 |
| B cells | $9.27 \pm 2.42$ | $40.5 \pm 10.5$ | **.0138** | **.0023** |
| $CD\ 86^+$ | $62.8 \pm 12.5$ | $84.9 \pm 5.73$ | .1358 | .0786 |
| $CD3^-CD11b^-CD200R^+$ | $11.4 \pm 1.78$ | $34.7 \pm 7.85$ | **.0134** | **.0022** |
| NK cells | $0.91 \pm 0.35$ | $0.61 \pm 0.24$ | .4894 | .3897 |
| Monocytes | $17.1 \pm 3.58$ | $15.1 \pm 1.93$ | .6295 | .1592 |
| $CD8^+$ | [b]$46.4 \pm 7.77$ | $34.9 \pm 7.91$ | .3234 | .8533 |
| $CD172a^+$ | $68.2 \pm 11.7$ | $53.6 \pm 9.22$ | .3467 | .5741 |
| $CD200R^+$ | $62.8 \pm 8.55$ | $75.9 \pm 3.87$ | .1870 | .0747 |
| **Large cells** | $94.6 \pm 1.26$ | $87.8 \pm 2.48$ | **.0293** | .1239 |
| Pulm macrophages | $97.3 \pm 0.59$ | $96.1 \pm 1.27$ | .4033 | .0856 |
| $CD172a^{Hi}$ | $96.1 \pm 0.77$ | $65.2 \pm 16.6$ | .0880 | $<$ **.0001**$^*$ |
| $CD172a^{Lo\ to\ -}$ | $0.89 \pm 0.22$ | $30.8 \pm 17.6$ | .1145 | $<$ **.0001**$^*$ |
| Granulocytes | [b]$1.33 \pm 0.34$ | $2.14 \pm 0.59$ | .2839 | .1962 |

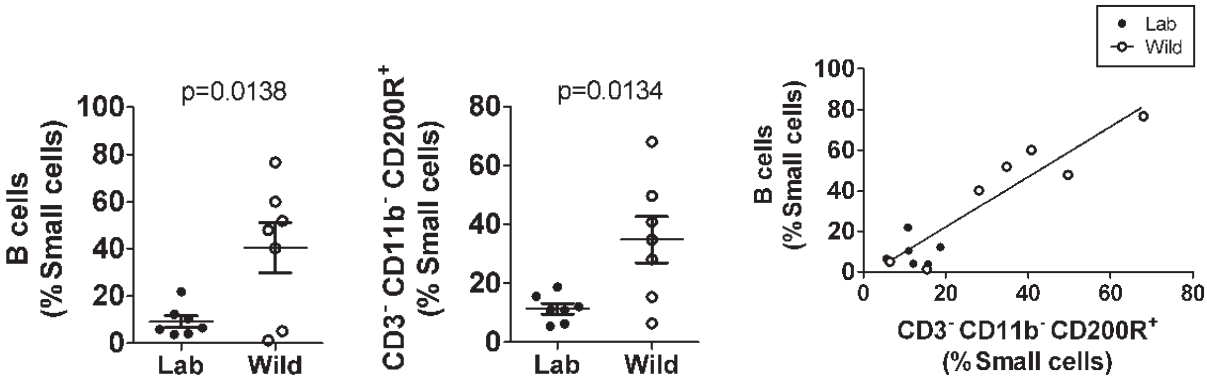
Small cells were defined as side $scatter^{Lo}$. T cells were defined as $CD3^+CD45RA^-$ and further phenotyped to T helper cells ($CD4^+CD8^-$), cytotoxic T cells ($CD4^-CD8^+$), double positive T cells ($CD4^+CD8^+$), and NK-T cells ($CD4^-CD8^+CD161^{Hi}$). B cells were defined as $CD3^-CD45RA^+MHCII^+$. NK cells were defined as $CD3^-CD8^+CD59^+CD161^{Hi}$. Monocytes were defined as $CD3^-CD11b^+$. Large cells were defined as side $scatter^{Hi}$. Pulmonary macrophages were defined as $CD3^-CD11b^+$. Granulocytes were defined as $CD3^-CD11b^+CD172^+HIS48^+$. The value given for all populations is expressed as a percentage of the parent population (e.g., the percentage of $FoxP3^+$ cells are reported as a percentage of $CD25^+$ cells, not helper T cells.) The means and standard errors are shown. Values that are significant at $P < .05$ are shown in **bold** and values that fit the criterion for $\alpha < 0.05$ following a Bonferroni correction ($P < .05/N = .0021$, with $N = 24$ for subpopulations of small cells and $P < .05/N = .0125$, with $N = 4$ for subpopulations of large cells) are marked by an asterisk $^*$. $N = 7$ lab rats and $N = 7$ wild-caught rats were used except where footnoted by [a] and [b]. Data from small cells and data from large cells were analyzed separately by ANOVA, and in both cases the difference between wild and laboratory rats was statistically significant.

[a] $N = 6$ wild-caught rats.

[b] $N = 6$ lab rats.

However, as shown in Figure 1, this feature was not found in 2 out of 7 of the wild rats analyzed, indicative of high variability in the immunology of the wild rats, despite the limited sample size. The finding that the lungs of wild rats tend to have more B cells was unexpected, meriting further study and highlighting the paucity of information regarding the state of immune systems in a "natural" environment. It was noted that a previously uncharacterized population of small cells ($CD3^-CD11b^-CD200R^+$) correlated strongly with the number of B cells in the wild rats (Figure 1), suggesting the possibility that the B cells were CD200R+. This possibility and the implications of this possibility deserve further study.

Immunoglobulin levels in the bronchoalveolar lavage fluid of wild and lab rats are shown in Figure 2 and in Table 2. Significant differences between wild and laboratory rats were seen in levels of IgE, IgM, and 3 of 4 subtypes of IgG. Interestingly, levels of IgG2b were not significantly different between the two groups, in contrast to previous results comparing immunoglobulin levels in the serum from wild and laboratory rats [18]. This might suggest differences between immunoglobulin production in the periphery versus in the lung. However, any negative result should be considered in light of the fact that the study design only identifies significant differences when the difference between wild and laboratory rats is approximately 1.2-fold




(a) (b) (c)

**Figure 1:** Relative numbers of (a) B cells and of (b) $CD3^{-}CD11b^{-}CD200R^{+}$ cells from the bronchoalveolar lavage fluid of laboratory and wild rats. The percentage of positive cells was determined by flow cytometry as described in Section 2. Datum for each animal as well as the mean values and standard errors are shown. The correlation between the levels of the two populations ($r^2 = .85$; $P = .003$) is shown in panel (c).

**Table 2:** Immunoglobulin levels in bronchoalveolar lavage fluid of laboratory and wild rats.

| | Lab rat (arbitrary units) | Wild rat (arbitrary units) | $P$-value mean | $P$-value variance |
|---|---|---|---|---|
| IgA | $0.442 \pm 0.097$ | $0.479 \pm 0.272$ | .8997 | .0240 |
| IgE | $0.034 \pm 0.007$ | $0.895 \pm 0.284$ | **.0104** | $<$ **.0001**$^*$ |
| IgM | $0.354 \pm 0.113$ | $0.860 \pm 0.098$ | **.0053**$^*$ | .7311 |
| IgG1 | $0.647 \pm 0.126$ | $2.049 \pm 0.379$ | **.0043**$^*$ | **.0168** |
| IgG2a | $0.653 \pm 0.117$ | $1.220 \pm 0.124$ | **.0061**$^*$ | .8937 |
| IgG2b | $1.320 \pm 0.198$ | $1.452 \pm 0.253$ | .6888 | .5634 |
| IgG2c | $0.265 \pm 0.031$ | $0.985 \pm 0.173$ | **.0015**$^*$ | **.0006**$^*$ |

Values were normalized by albumin content. The means and standard errors are shown. Values that are significant at $P < .05$ are shown in **bold** and values that fit the criterion for $\alpha < .05$ following a Bonferroni correction ($P < .05/N = 0.0071$, with $N = 7$) are marked by an asterisk $^*$. $N = 7$ lab rats and $N = 7$ wild-caught rats were used. Analysis by ANOVA showed that the difference between wild and laboratory rats was significant ($P < .0001$).

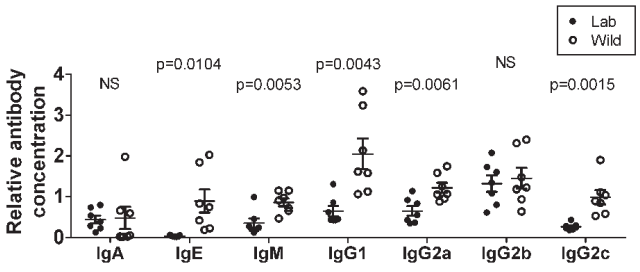



**Figure 2:** Relative immunoglobulin levels in the bronchoalveolar lavage fluid of laboratory and wild rats. The antibody levels were determined by ELISA as described in Section 2. Datum for each animal as well as the mean values and standard errors are shown. NS = not statistically significant ($P > .05$).

greater than the average standard deviation of the two groups (see Section 2). On the other hand, even with the limited number of animals used, it is readily evident that IgG2b production in wild and laboratory rats is relatively similar in the lung, and that the range of IgG2b production in the two groups of animals strongly overlaps. Thus, if further analysis of more animals does reveal statistically significant differences, these differences are expected to be much less profound than differences in the production of other antibodies in the lung, such as IgE and IgG2c, for which no overlap in range between the two groups was observed (Figure 2).

### 3.3 T cell phenotypes in the bronchoalveolar lavage fluid of wild and laboratory rats

Evaluation of markers on the T cells from the bronchoalveolar lavage fluid of wild and lab rats revealed statistically significant differences between expression of several molecules that are indicative of fundamental properties of the T cells, including reactivity and maturity (Table 1 and Figure 3). For example, the average level of MHCII on T cells was significantly higher in wild rats than in lab rats, whereas levels of CD62L tended to be lower on T cells. Similar findings, previously reported in the periphery of wild and lab rats [45] suggest that at least some key differences in T cell populations between wild and laboratory rats are widespread, independent of the tissue examined.

**Table 3:** Cytokine and chemokine levels in bronchoalveolar lavage fluid of laboratory and wild rats.

| | Lab rat (pg/mL) | Wild rat (pg/mL) | $P$-value mean | $P$-value variance |
|---|---|---|---|---|
| IL-1$\alpha$ | $3.19 \pm 1.28$ | $2.36 \pm 1.82$ | .7232 | .3261 |
| IL-1$\beta$ | $6.90 \pm 1.53$ | $11.7 \pm 2.45$ | .1302 | .2103 |
| IL-2 | $5.08 \pm 0.67$ | $2.68 \pm 0.31$ | **.0048** | .0942 |
| IL-4 | $0.25 \pm 0.03$ | $0.27 \pm 0.03$ | .6353 | .7254 |
| IL-5 | $22.2 \pm 0.59$ | $16.5 \pm 0.83$ | **.0001**$^*$ | .3507 |
| IL-6 | $20.9 \pm 3.24$ | $16.0 \pm 3.69$ | .3506 | .6478 |
| IL-10 | $1.31 \pm 0.17$ | $0.92 \pm 0.23$ | .2039 | .3822 |
| IL-12($p$40) | $5.27 \pm 1.53$ | $6.63 \pm 2.21$ | .6325 | .3118 |
| IL-12($p$70) | $7.03 \pm 0.62$ | $4.39 \pm 0.60$ | **.0090** | .9783 |
| IL-13 | $5.71 \pm 0.71$ | $5.48 \pm 0.98$ | .8553 | .3519 |
| IL-17 | $1.61 \pm 0.10$ | $1.18 \pm 0.50$ | .6945 | .1450 |
| CCL2 (MCP-1) | $57.4 \pm 16.1$ | $38.2 \pm 8.08$ | .2877 | .1271 |
| CCL3 (MIP-1$\alpha$) | $7.17 \pm 1.38$ | $4.83 \pm 1.03$ | .1893 | .5665 |
| CCL5 (RANTES) | $13.8 \pm 1.37$ | $11.4 \pm 0.77$ | .1360 | .2052 |
| CCL7 (MCP-3) | $15.1 \pm 6.56$ | $14.1 \pm 3.62$ | .8877 | .1926 |
| CCL11 (Eotaxin) | $7.65 \pm 2.03$ | $19.0 \pm 3.27$ | **.0137** | .2070 |
| CXCL1 (Gro-$\alpha$) | $1.41 \pm 0.44$ | $1.28 \pm 0.81$ | .8963 | .1224 |
| CXCL2 (MIP-2) | $37.9 \pm 12.4$ | $21.7 \pm 6.04$ | .2418 | .1102 |
| CXCL10 (IP-10) | $16.3 \pm 1.82$ | $17.4 \pm 2.26$ | .7090 | .5113 |
| G-CSF | $0.86 \pm 0.14$ | $0.65 \pm 0.18$ | .3839 | .4666 |
| GM-CSF | $1.20 \pm 0.64$ | $0.22 \pm 0.15$ | .1347 | **.0024** |
| ICAM | $11,515 \pm 2873$ | $2,137 \pm 248$ | **.0040** | $<$ **.0001**$^*$ |
| IFN-$\gamma$ | $5.24 \pm 0.65$ | $3.04 \pm 0.47$ | **.0153** | .5295 |
| Leptin | $29.4 \pm 2.58$ | $15.8 \pm 2.98$ | **.0046** | .6248 |
| NGF | $5.28 \pm 1.32$ | $5.74 \pm 1.57$ | .8266 | .5709 |
| sRANKL | $4.05 \pm 0.49$ | $2.59 \pm 0.75$ | .1369 | .2440 |
| TGF-$\beta$1 | $34.0 \pm 12.2$ | $32.0 \pm 5.54$ | .8749 | .0814 |
| TGF-$\beta$2 | $4,562 \pm 380$ | $1,155 \pm 285$ | $<$ **.0001**$^*$ | .5782 |
| TGF-$\beta$3 | $23.2 \pm 1.91$ | $15.3 \pm 1.44$ | .0051 | .5823 |
| TNF-$\alpha$ | $7.38 \pm 1.68$ | $4.39 \pm 0.68$ | .1061 | **.0469** |
| VEGF | $434 \pm 70$ | $150 \pm 36$ | **.0025** | **.1484** |
| Albumin | $31,800 \pm 8,752$ | [a]$27,300 \pm 3,863$ | .6662 | .0686 |

The means and standard errors are shown. Values that are significant at $P < .05$ are shown in **bold** and values that fit the criterion for $\alpha < 0.05$ following a Bonferroni correction ($P < .05/N = .0016$, with $N = 31$) are marked by an asterisk $^*$. $N = 7$ lab rats and $N = 8$ wild-caught rats were used except where footnoted by [a]. Analysis by ANOVA showed that the difference between wild and laboratory rats was significant ($P < .0001$).
[a]$N = 7$ wild-caught rats.

### 3.4 Cytokine and chemokine levels in the bronchoalveolar lavage fluid of wild and laboratory rats

Several indicators in the bronchoalveolar lavage fluid suggested that laboratory rats have substantially less adaptive immune activation than do the laboratory rats tested (Table 3 and Figure 4). Levels of IL-2, generally associated with T cell stimulation, and IL-5, associated with allergic responses, were significantly higher in laboratory rats. However, TGF-$\beta$2, involved in fibrotic responses, and soluble ICAM, which down-regulates immune responses were also elevated in the laboratory rodents compared to the wild rodents (Table 3 and Figure 4).

### 3.5 Monocytes and granulocytes in the periphery and spleen of wild and laboratory rats

As shown in Figure 5, wild-caught rats had, on average, a much greater representation of monocytes as a fraction of total leukocytes in their blood than did the lab rats. Our study identified that an average of 11% of the leukocytes were monocytes in the periphery of the laboratory rats, versus about 20% in the wild rats (Table 4). A review of the literature corroborates the observation that the levels of monocytes in laboratory rats is relatively low, estimated to be 0 to 5% [3], 2–3% [21], and 10% [27] of the total leukocytes, depending on the study. Thus, the literature

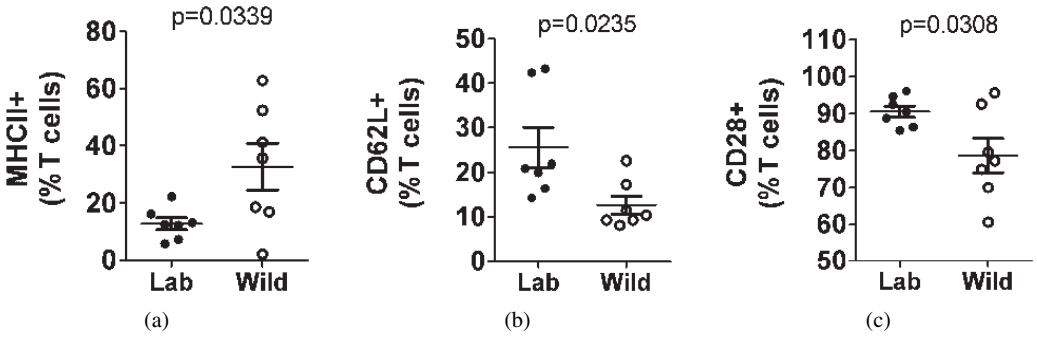



**Figure 3:** Phenotypes of T cells from bronchoalveolar lavage fluid of laboratory and wild rats. The percentages of cells positive for (a) MHCII, (b) CD62L, and (c) CD28 were determined by flow cytometry as described in Section 2. Datum for each animal as well as the mean values and standard errors are shown.

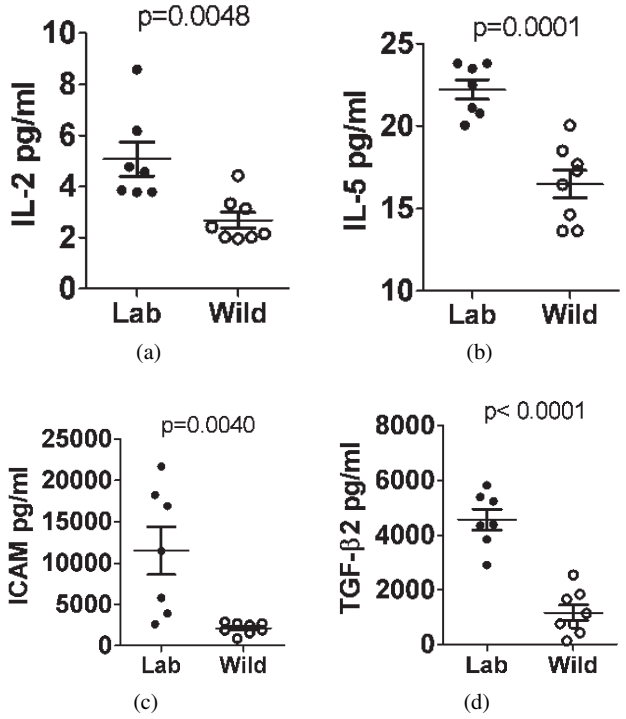



**Figure 4:** Cytokine and chemokine levels in the bronchoalveolar lavage fluid of laboratory and wild rats. Levels of (a) IL-2, (b) IL-5, (c) ICAM, and (d) TGF-$\beta$2 were determined using multiplex suspension arrays as described in Section 2. Datum for each animal as well as the mean values and standard errors are shown.

is consistent with the idea that laboratory rats have lower levels of peripheral monocytes than do the wild rats used in this study. This difference amounted to greater than 70% more monocytes as a percent of total immune cells in the wild rats (Table 4). This difference was not observed in the spleen (Table 5), confirming a previous report [29] finding no statistically significant differences in the immune cell populations in the spleens of wild-caught rats versus lab rats.

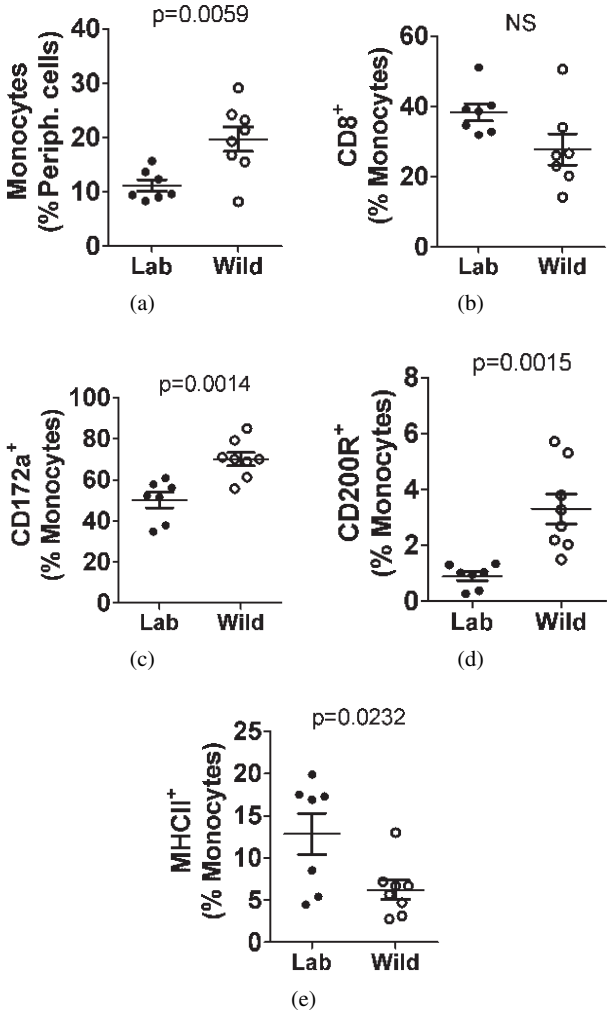



**Figure 5:** Phenotypes of peripheral monocytes in laboratory and wild rats. The (a) percentage monocytes and the percentage of monocytes positive for (b) CD8, (c) CD172a, (d) CD200R, and (e) MHCII were determined by flow cytometry as described in Section 2. Datum for each animal as well as the mean values and standard errors are shown. NS = not statistically significant ($P > .05$).

**Table 4:** Phenotypes of monocytes and granulocytes cells from peripheral blood of laboratory and wild rats.

| Cell population | Lab rat (%) | Wild rat (%) | $P$-value mean | $P$-value variance |
|---|---|---|---|---|
| Monocytes | $11.2 \pm 1.01$ | $19.7 \pm 2.25$ | **.0059** | .0609 |
| $CD8^+$ | $38.3 \pm 2.5$ | [a]$27.8 \pm 4.44$ | .0601 | .1769 |
| $CD172a^+$ | $50.3 \pm 3.79$ | $70.2 \pm 3.24$ | **.0014**$^*$ | .8122 |
| $CD200R^+$ | $0.90 \pm 0.16$ | $3.31 \pm 0.54$ | **.0015**$^*$ | **.0055**$^*$ |
| $MHCII^+$ | $12.9 \pm 2.45$ | $6.23 \pm 1.13$ | **.0232** | .0869 |
| Granulocytes | $8.11 \pm 1.97$ | $16.0 \pm 4.64$ | .1608 | **.0389** |
| Eosinophils | $39.0 \pm 8.63$ | $11.5 \pm 2.85$ | **.0069** | **.0147** |
| Basophils | $1.11 \pm 0.91$ | $16.3 \pm 7.94$ | .1001 | $<$ **.0001**$^*$ |
| [b]$bCD59^+$ | $83.2 \pm 6.87$ | $99.5 \pm .08$ | **.0244** | $<$ **.0001**$^*$ |
| [b]$bCD62L^+$ | $92.0 \pm 1.92$ | $77.6 \pm 8.10$ | .1298 | **.0017**$^*$ |
| [b]$bCD90^+$ | $33.2 \pm 4.84$ | $70.4 \pm 8.48$ | **.0029**$^*$ | .1475 |
| [b]$bCD161a^+$ | $72.4 \pm 9.93$ | $29.9 \pm 7.80$ | **.0046**$^*$ | .6545 |
| $Neutrophils^+$ | $52.1 \pm 7.39$ | $64.9 \pm 10.6$ | .3527 | .3124 |
| $MHCII^+$ | $8.62 \pm 1.39$ | $6.79 \pm 1.95$ | .4694 | .3436 |

Monocytes were defined as $CD3^-CD4^+CD11b^+$. Granulocytes were defined as $CD3^-CD11b^+HIS48^+$. Eosinophils, basophils, and neutrophils were separated by forward and side scatter properties of granulocyte populations. The value given for all populations is expressed as a percentage of the parent population. Values that are significant at $P < .05$ are shown in **bold** and values that fit the criterion for $\alpha < .05$ following a Bonferroni correction ($P < .05/N = .0125$, with $N = 4$ for subpopulations of monocytes and $P < .05/N = .0065$, with $N = 8$ for subpopulations of granulocytes) are marked by an asterisk $^*$. $N = 7$ lab rats and $N = 8$ wild-caught rats were used except where footnoted by [a]. Data from monocytes and granulocytes were analyzed separately by ANOVA, and the difference between wild and laboratory rats was significant ($P < .0001$).
[a]$N = 7$ wild-caught rats.
[b]$CD59^+$, $CD62L^+$, $CD90^+$, and $CD161a^+$ are given as a percentage of eosinophils and basophils combined.

**Table 5:** Phenotypes of monocytes and granulocytes cells from spleens of laboratory and wild rats.

| Cell population | Lab rat (%) | Wild rat (%) | $P$-value mean | $P$-value variance |
|---|---|---|---|---|
| Monocytes | $7.84 \pm 1.36$ | $10.1 \pm 0.88$ | .1672 | .4688 |
| $CD8^+$ | $14.9 \pm 2.11$ | $9.74 \pm 1.03$ | **.0347** | .1662 |
| $CD172a^+$ | $19.8 \pm 3.75$ | $34.7 \pm 2.78$ | **.0068**$^*$ | .6840 |
| $CD200R^+$ | $49.8 \pm 5.75$ | $54.9 \pm 3.65$ | .4525 | .4418 |
| $MHCII^+$ | $51.3 \pm 3.51$ | $49.4 \pm 5.62$ | .7945 | .1942 |
| Granulocytes | $8.51 \pm 1.06$ | $15.3 \pm 2.58$ | .0523 | **.0365** |
| Eosinophils | $68.9 \pm 13.0$ | $50.9 \pm 10.8$ | .3050 | .8786 |
| Basophils | $13.0 \pm 12.0$ | $31.6 \pm 9.27$ | .2367 | .7522 |
| [a]$aCD59^+$ | $90.9 \pm 1.73$ | $99.4 \pm .012$ | $<$ **.0001**$^*$ | $<$ **.0001**$^*$ |
| [a]$aCD62L^+$ | $45.0 \pm 7.55$ | $46.4 \pm 7.59$ | .8998 | .7660 |
| [a]$aCD90^+$ | $60.1 \pm 8.78$ | $74.4 \pm 4.70$ | .1491 | .2414 |
| [a]$aCD161a^+$ | $81.2 \pm 14.2$ | $41.2 \pm 8.19$ | **.0236** | .3194 |
| Neutrophils | $11.8 \pm 1.56$ | $10.3 \pm 1.32$ | .4551 | .9151 |
| $MHCII^+$ | $13.6 \pm 2.23$ | $10.7 \pm 3.16$ | .5040 | .2937 |

Monocytes were defined as $CD3^-CD4^+CD11b^+$. Granulocytes were defined as $CD3^-CD11b^+CD172^+HIS48^+$. Eosinophils, basophils, and neutrophils were separated by forward and side scatter properties of granulocyte populations. The value given for all populations is expressed as a percentage of the parent population except where noted in footnote [a]. Values that are significant at $P < .05$ are shown in **bold** and values that fit the criterion for $\alpha < .05$ following a Bonferroni correction ($P < .05/N = .0125$, with $N = 4$ for subpopulations of monocytes and $P < .05/N = .0065$, with $N = 8$ for subpopulations of granulocytes) are marked by an asterisk $^*$. $N = 6$ lab rats and $N = 8$ wild-caught rats were used. Data from monocytes and granulocytes were analyzed separately by ANOVA, and only the difference in granulocyte phenotypes between wild and laboratory rats was significant ($P = .008$).
[a]$CD59^+$, $CD62L^+$, $CD90^+$ and $CD161a^+$ are given as a percentage of eosinophils and basophils combined.

One of the hallmarks of parasitic infection in laboratory animals is a profound increase in granulocytes, particularly eosinophils and basophils. While the average amount of granulocytes in wild rats was about double (as a percent of total leukocytes) that found in laboratory rats in both the periphery (Table 4 and Figure 6) and the spleen (Table 5), the range of the data overlapped significantly between the two populations, and the difference between the two types of rats was not significantly different (Tables 4 and 5, Figure 6). Further, the type of granulocyte (i.e., eosinophil, basophil, or neutrophil) varied widely in the periphery and in the spleen, with, unexpectedly, the only statistically significant difference between wild and laboratory rats being less eosinophils as a percent of total granulocytes in the periphery of wild

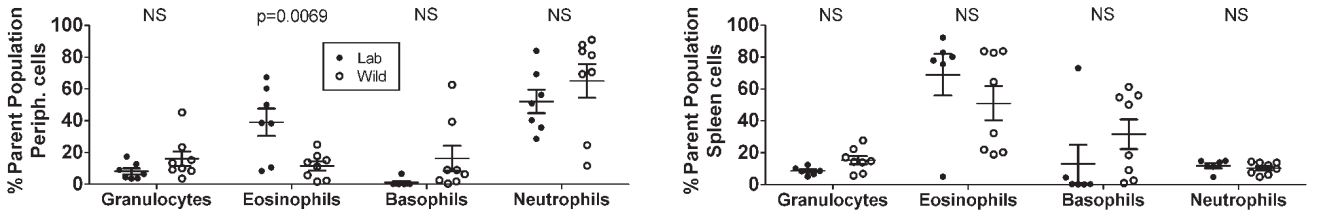



**Figure 6:** Phenotypes of (a) peripheral and (b) spleenic granulocytes in laboratory and wild rats. The percentage of various granulocyte types was determined by flow cytometry as described in Section 2. Datum for each animal as well as the mean values and standard errors are shown. NS = not statistically significant ($P > .05$).

rats (Table 4). A survey of the literature shows that the percentage of leukocytes identified as granulocytes in laboratory rats is somewhat variable, perhaps depending on genetics, gender, techniques used, and other factors. Estimates of the number of granulocytes as a percent of total leukocytes in the peripheral blood of rats range from 9% to 40% [3,28].

Although differences between the number and type of granulocytes between wild and laboratory rats were not impressive, several markers, including CD59, CD90, and CD161a, were expressed differently between the basophils of wild and laboratory rats (Tables 4 and 5). Similar observations have been made when evaluating lymphocyte populations in wild and laboratory rats, particularly in the spleen [45], where the phenotype of particular cell populations more so than the relative ratios of particular cell populations appears to be strongly influenced by wild versus laboratory conditions.

These data further strengthen the concept that the ubiquitous responses of laboratory animals experimentally exposed to environmental stimuli (e.g., increased production of regulatory T cells and granulocytes after exposure to helminths) are not necessarily similar to the steady state immune systems of animals born and raised in the presence of those same stimuli.

### 3.6 Antigen expression on peripheral and spleenic monocytes

As noted above, wild rats had greater than 70% more monocytes as a percent of total immune cells in the periphery than did the lab rats used in this study (Figure 5 and Table 4). However, the CD8 expression on those monocytes was not significantly different in the two rat populations, possibly suggesting no substantial differences in levels of monocytes activation between the two populations [23,31, 40,41]. However, wild rat monocytes expressed, on average, significantly more CD172a than did lab rat monocytes (Figure 5 and Table 4). Also known as signal regulatory protein alpha, CD172a is known to modulate cells of the innate immune system [13,30]. When properly engaged, CD172a prevents phagocytosis by alveolar macrophages, and can prevent production of TNF-$\alpha$ by monocytes [26, 43]. Thus, an increased expression of this marker might suggest yet another mechanism by which systems with a biome unmanipulated by modern medicine and sanitation are less prone to inflammatory responses. However, this trend toward greater expression of CD172a on wild rat monocytes was not observed in monocytes found in the bronchoalveolar lavage fluid (Table 1).

Of interest was the observation that OX-2 receptor (CD200R) expression on wild rat peripheral monocytes, while not expressed by more than 10% of the monocytes in any animal evaluated in this study, was expressed on an average of 3-fold more in wild rat peripheral monocytes than in lab rat monocytes (Table 4 and Figure 5). This receptor is involved in the OX-2-mediated T cell costimulatory pathway, which is functionally independent of the B7/CD28 pathway [12]. Blockade of the OX-2/OX-2 receptor interaction exacerbates the disease model of experimental allergic encephalomyelitis in Lewis rats [47], indicating that the OX2/OX-2 receptor interaction is important in mediating immune function. Interestingly, this trend toward dramatically greater expression of CD200R on wild rat monocytes was not observed in monocytes found in the bronchoalveolar lavage fluid (Table 1) or in the spleen (Table 5).

### 3.7 Innate immune-related cytokine and chemokine levels in the serum of wild and laboratory rats

A comparison between the lymphocyte-related cytokine and chemokine levels in the sera of wild and laboratory rats has previously been published [45]. Table 6 shows a comparison between innate immune-related cytokines and chemokines in the sera of wild and laboratory rats. With the exception of leptin, the concentration of all analytes showed a trend toward a difference, with 6 out of 9 showing a significant difference ($P < .05$) and 4 out of 9 showing a significant difference following the Bonferroni correction (Table 6 and Figure 7). Increased levels of G-CSF and GM-CSF have been associated with inflammatory responses to chronic infections in patients with cystic fibrosis [33], and increased concentrations of these growth factors in the laboratory rats suggest increased levels of baseline innate immune activation in the those animals compared to their wild counterparts.

**Table 6:** Cytokine and chemokine levels in sera of laboratory and wild rats.

| | Lab rat (pg/mL) | Wild rat (pg/mL) | $P$-value mean | $P$-value variance |
|---|---|---|---|---|
| CCL2 (MCP-1) | $755 \pm 75.4$ | $1754 \pm 255$ | **.0037**$^*$ | **.0059** |
| CCL3 (MIP-1$\alpha$) | $124 \pm 10.2$ | $80.7 \pm 13.6$ | **.0308** | .4412 |
| CCL7 (MCP-3) | $448 \pm 41.1$ | $616 \pm 68.6$ | .0635 | .1788 |
| CCL11 (Eotaxin) | $100 \pm 5.35$ | $123 \pm 12.6$ | .1414 | .0580 |
| [a]CXCL1 (Gro-$\alpha$) | $80.6 \pm 11.2$ | $38.6 \pm 5.66$ | **.0050**$^*$ | .1709 |
| [a]G-CSF | $95.0 \pm 7.25$ | $34.8 \pm 7.60$ | **.0001**$^*$ | .8028 |
| [a]GM-CSF | $27.4 \pm 1.56$ | $18.8 \pm 2.77$ | **.0254** | .1721 |
| Leptin | $55.9 \pm 20.1$ | $73.0 \pm 56.9$ | .7933 | **.0154** |
| [a]VEGF | $74.1 \pm 7.66$ | $27.4 \pm 4.26$ | **.0002**$^*$ | .2476 |

The means and standard errors are shown. Values that are significant at $P < .05$ are shown in **bold** and values that fit the criterion for $\alpha < .05$ following a Bonferroni correction ($P < .05/N = .0056$, with $N = 9$) are marked by an asterisk $^*$. $N = 7$ lab rats and $N = 8$ wild-caught rats were used except where footnoted by [a]. Analysis by ANOVA showed that the difference between wild and laboratory rats was significant ($P < .0001$).
[a]$N = 6$ lab rats and $N = 7$ wild-caught rats.

The lack of a profound difference in leptin (a hormone that has a central role in fat metabolism) expression in wild versus laboratory rodents (Table 6) has precedence. Previous work in mice [1] has shown a statistically significant difference between serum leptin concentrations in wild and laboratory animals, but the concentration of this analyte overlapped extensively between individual wild and laboratory rats (concentrations for wild and laboratory mice of $4.57 \pm 3.00$ and $2.61 \pm 1.75$ ng/mL (mean $\pm$ SD, resp.)), and a relatively large cohort of animals ($> 60$) was used in order to clearly define the differences between wild and laboratory mice. These results are consistent with the idea that this particular marker is less perturbed by laboratory conditions than the innate immune-related markers evaluated in the present study, and adds to questions regarding a substantial effect of laboratory conditions on the fat metabolism of rodents. On the other hand, the amount of leptin in the bronchoalveolar lavage fluid was significantly different between wild and laboratory rodents (Table 1). Interestingly, the average amount of leptin was almost twice as high in the bronchoalveolar lavage fluid of laboratory rats, in contrast to the results obtained using the sera of mice [1], where wild mice had more than laboratory animals. This finding illustrates the necessity of examining various tissues independently, and draws attention to the complex differences between wild and laboratory rodents.

### 3.8 Expression indices as a measure of immune system activity

Despite many statistically significant differences in the average immune parameters between wild and laboratory rats, there was usually some overlap in the range of data between the two groups (Figures 1–7). This observation suggests that either some wild rats were in fact similar to their laboratory counterparts, or perhaps that the two groups are clearly distinct, but that multiple parameters must be assessed in order to appreciate that distinction. To probe this idea, a composite, or "expression index" of the data shown in Figures 1, 3, 4, 5, and 7 was created. Data from immunoglobulin levels in the bronchoalveolar lavage fluid (Figure 2) were not used because levels of IgE and IgG subtype were very strongly correlated with the type of rat and may have heavily influenced the index. Further, data regarding the phenotype of granulocyte in the periphery (Figure 6) were not used, since this parameter was not apparently strongly influenced by the type of rat.

To combine results into expression indices, data that were statistically significant in these figures (Figures 1, 3, 4, 5, and 7) were normalized such that the mean for all parameters in the rats (wild and lab rats combined) was 1.0. Further, the data were adjusted such that the mean for the laboratory rats was always greater (an arbitrary assignment) than that of the wild rats for each parameter measured. In cases where the mean for the wild rats was greater, all data for that parameter were "inverted" using $(-x + 2)$, where $x$ is the normalized value.

As shown in Figure 8, the range of expression indices for phenotypes of immune cells in the bronchoalveolar lavage fluid (Figure 8(a)) overlapped between wild and laboratory rats, with two wild rats exhibiting properties similar to the laboratory rats. However, the ranges of cytokine and chemochine expression in the bronchoalveolar lavage fluid (Figure 8(b)), phenotypes of innate immune cells in the periphery (Figure 8(c)), and innate immune-related cytokine and chemochine expression in the periphery (Figure 8(d)) showed clear separations between wild and laboratory rats. Further, the overall composite expression index (Figure 8(e)) showed an even greater separation between the ranges of data for wild and laboratory rats. Thus, although particular immune parameters may be useful in assessing the immune function, with perhaps some exceptions (MHCII expression on T cells or the amount

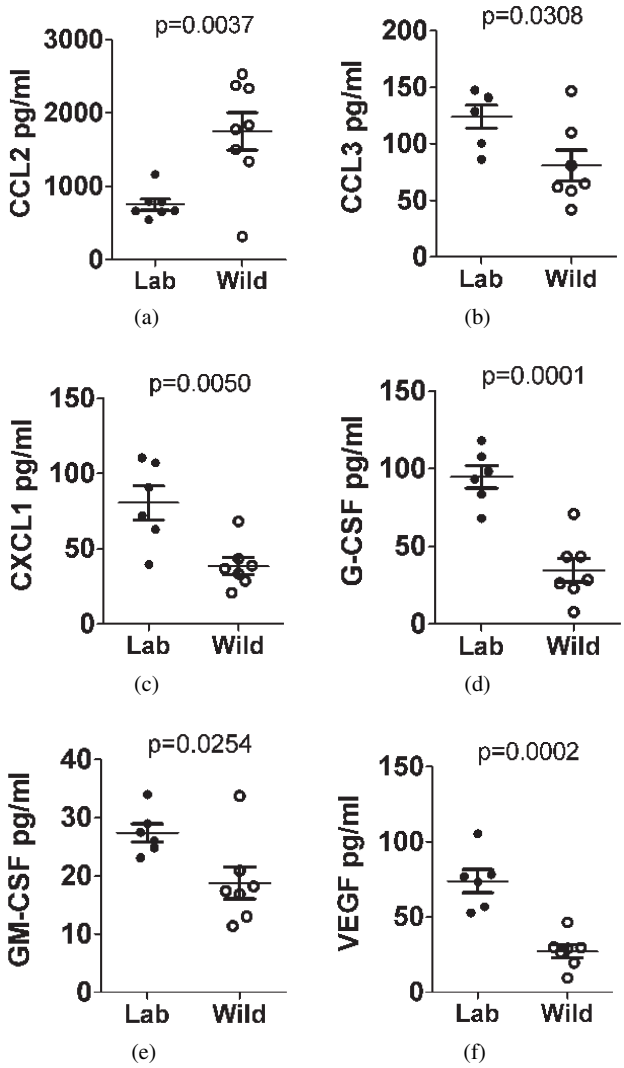



**Figure 7:** Innate immune-related cytokine and chemokine levels in the serum of laboratory and wild rats. Levels of (a) CCL2, (b) CXCL1, (c) G-CSF, (d) GM-CSF, and (e) VEGF were determined using multiplex suspension arrays as described in Section 2. Datum for each animal as well as the mean values and standard errors are shown.

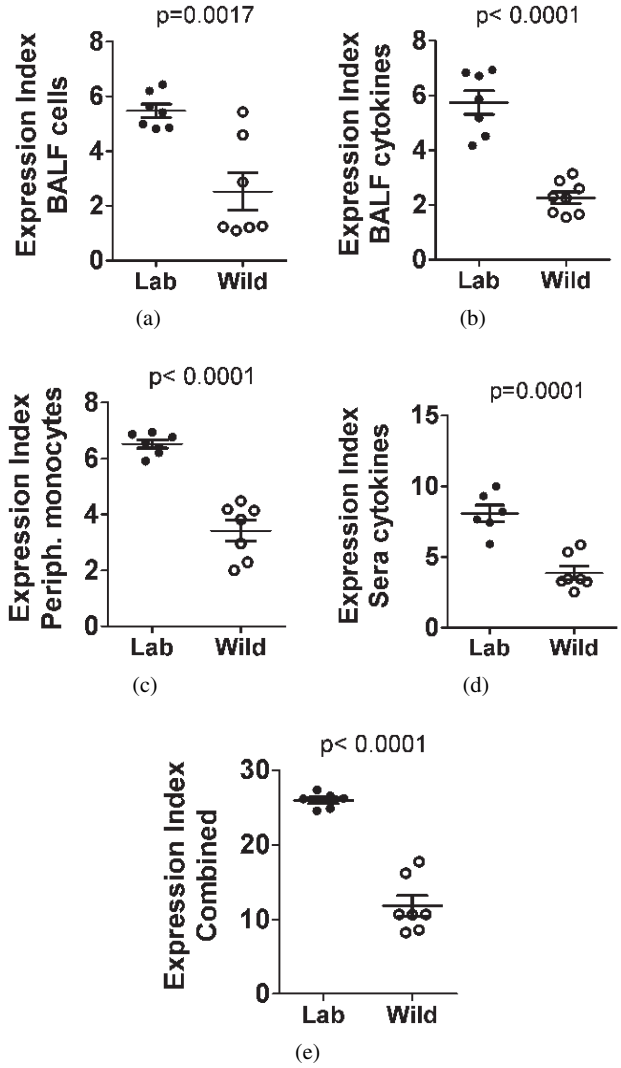



**Figure 8:** Expression indices derived from all statistically significant comparisons ($P < .05$) shown in (a) Figures 1 and 3, (b) Figure 4, (c) Figure 5, and (d) Figure 7. The expression indices were calculated as described in Section 3.8, and provide a general assessment of immune activity, with the laboratory rats arbitrarily set as having the higher index. Data in Figures 1(a) and 1(b) were highly correlated, so only data from Figure 1(a) were used.

of IgE production), it is a composite of several parameters which seems to be most telling with regard to immune function. These data further suggest that all wild-caught individuals in the sample studied are clearly distinct from all lab rats studied, even though the analysis of individual immune parameters alone shows a substantial overlap.

Of considerable interest was the narrow range of expression indices covered by the laboratory rats (Figure 8(e)). The standard deviation for the wild rats was more than 3-fold greater than that of the laboratory rats (F test to compare variances; $P = .015$), suggesting that the laboratory rats used in this study might have a much narrower range of immune activity than do the wild rats.

## 4 Discussion

Wild rodents, unlike their laboratory counterparts today, are exposed to a wide range of infectious agents [19,46] that include macro- (helminths and parasitic arthropods) and microparasites (viruses, bacteria, protozoa, and fungi). Indeed, many of these organisms were identified in the wild rats evaluated in this study. In contrast, recent changes in the housing of most laboratory rats have depleted these animals of virtually all of their co-evolved helminths and other parasitic organisms [2,36]. Thus, wild-caught rodents can serve as a model for humans with biomes unmanipulated

by modern medicine and sanitation practices, whereas laboratory rodents can serve as a model for humans living with depleted biomes in post-industrial cultures.

Some of the factors other than biome depletion that are altered by changing culture have been discussed previously [45]. These factors, affecting both humans and rodents, include exercise, stress, and nutrition, and all play a widely recognized role in modulating the immune system. Excess stress, for example, has been associated with autoimmune disease [44]. Thus, it remains unknown to what extent changes in the biome versus other changes associated with domestication or post-industrial culture affect the immune system. Further, although the wild/laboratory rat model we utilize has provided a wealth of information, it has limitations in that it cannot separate the roles of biome depletion in immune dysregulation from the influence of other environmental factors or of genetics. The role of genetics in particular demands a further study, and a critical next step must be the evaluation of immune variability in biome depleted and biome normal animals that have similar genetic profiles. However, a wide range of evidence, from the fields of evolutionary biology, ecology, immunology, medicine, and biomedical research point toward biome depletion as the single most influential factor affecting epidemics of immune related disease in post industrial culture [9,38].

The data presented herein add further to the list of immune parameters that differ between wild and laboratory rats. As such, this study adds to the ever increasing understanding of the fundamental nature of a "normal" immune system, one that exists in the "environment of evolutionary adaptedness," with a biome unmanipulated by modern medicine and sanitation practices. However, given the limited number of animals we have studied to date and the ever increasing understanding of various immune components, further studies are certainly mandated. Indeed, as pointed out in in Sections 1 and 2, the study is designed to identify major differences in a broad array of immune parameters, not to identify relatively small differences or to sort out factors which affect immunity in the wild. At the same time, these studies point strongly toward the limitations and potential pitfalls of evaluating a small number of immune parameters within selected compartments in wild animals: a broad survey may be necessary to capture a reasonable picture of immunity in wild animals. At the same time, studies examining fewer parameters with larger sample sizes will undoubtedly prove useful in the assessment of factors which affect specific parameters.

One of the most striking features observed between the wild and laboratory rats we evaluated was the increased average levels of GM-CSF and G-CSF in the blood of the laboratory rats compared to the wild rats. Average levels of GM-CSF and G-CSF were 46% and 173% greater, respectively, in the serum of the laboratory rats. These two factors are important for the proliferation and differentiation of hematopoietic progenitor cells into granulocytes, neutrophils, and macrophages. In addition, GM-CSF is a strong chemoattractant for neutrophils, and enhances the cytotoxic activity of neutrophils and macrophages [14]. Elevated levels of these molecules might certainly account for increased levels of immune responsiveness in laboratory rats. The presence of elevated levels of CXCL-1, another factor involved in chemotaxis and cell activation of neutrophils, in the sera of laboratory rats may have similar effects. Of interest is the observation that the ratio of GM-CSF and G-CSF can serve as an indicator of the immune system's balance between TH1 and TH2 responses [33]. The observation that average levels of both GM-CSF and G-CSF are increased in the serum of lab rats might suggest that it is the gross level of immune reactivity rather than an imbalance favoring a specific activity that is the primary hallmark of the laboratory rat immunity. This idea is consistent with the observation that Western culture is associated with increased levels of both allergy and autoimmune disease in humans.

Previous studies from our laboratory have identified dozens of differences between the typical wild rat-derived immune system and that of the laboratory rat [18,29,45]. However, despite the limited number of animals that have been evaluated to date, relatively few indicators of immunity have been identified which differ consistently between all wild rats and all laboratory rats: the ranges of immune parameters usually overlap between the two populations, often because of high variability in the wild rat-derived data. Further, differences between wild and laboratory animals in one tissue do not necessarily correspond to differences in another tissue. For example, examining the same animals evaluated in this manuscript, the expression of MHCII on the peripheral T cells was previously found to be higher in all wild rats compared to all laboratory rats [45]. However, this distinction found in the periphery is lost in the lung, where some overlap between wild and laboratory rats is observed (Figure 1). In other words, while the immune system of all wild rats seems to be different from that of all laboratory rats, as indicated by the "expression indices" described in the results, there are few specific features of the wild-derived immune system which are found in all individuals, and a wide range of variability in the wild rodents seems to be the norm. Future work evaluating greater numbers of wild animals may reveal substantially more variability, and might perhaps be utilized to identify some of the sources of the variability.

The high degree of variability in the wild-derived immune system is encouraging because it points toward the idea that biome reconstitution to avoid disease may not have very precise requirements. Consistent with this idea is the observation that different human populations living

in pre-industrial societies have constitutionally different biomes dependent on diet, social behavior, water supply, climate, and other factors. Yet, none of the post-industrial-associated allergic and autoimmune diseases are known to plague any pre-industrial culture. Perhaps more telling is the observation that colonization by any one of several helminths was sufficient to halt the progression of multiple sclerosis [16, 15], a disease which has largely eluded the best efforts of modern medicine.

The fact that we live in the Information Age but have immune systems adapted for the Stone Age underlies a wide range of illnesses, and provides a great challenge to the field of clinical immunology. The challenge seems to have reached a crisis level, with epidemics of immune related illnesses in the population thwarting attempts at a cure [17]. Further, an ever increasing appreciation for the vast complexity of the immune system and of the changes induced by biome depletion reinforce doubts that modern medicine has the capacity to redirect the wayward immune system using pharmaceutical approaches. However, at the same time, there is a cause for hope for several reasons. The idea that biome depletion underlies epidemics of immune disease indicates that restoration of the biome, which is an extremely feasible prospect [9], is likely to stem the tide of disease. Further, the data presented herein, in conjunction with other observations described above, point toward the idea that reconstitution of the human biome may not consist of a precise regimen, but rather may be achieved by a wide range of potential therapies.

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